Accepted Manuscript

Enhanced formation of Giant cells in common variable immunodeficiency; relation to granulomatous disease

Timothy H. Scott-Taylor, Karen Whiting, Ruth Pettengell, David A. Webster

PII: S1521-6616(16)30591-5
Reference: YCLIM 7762

To appear in: Clinical Immunology

Received date: 21 February 2016
Revised date: 17 October 2016
Accepted date: 11 November 2016

Please cite this article as: Timothy H. Scott-Taylor, Karen Whiting, Ruth Pettengell, David A. Webster, Enhanced formation of Giant cells in common variable immunodeficiency; relation to granulomatous disease, Clinical Immunology (2016), doi: 10.1016/j.clim.2016.11.002

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
Enhanced Formation of Giant Cells in Common Variable Immunodeficiency; Relation to Granulomatous Disease

Timothy H. Scott-Taylor¹, Karen Whiting², Ruth Pettengell³, David A. Webster¹ .

¹Department of Immunology, University College London, Royal Free Hospital NHS Trust, Pond Street, Hampstead, London NW3 2QG
²Faculty of Sciences, Kingston University, Penrhyn Road, Kingston upon Thames, Surrey, KT1 2EE
³Department of Haematology, St George’s University of London, Cranmer Terrace, London SW17 0RE

Running Title: Giant cells in CVID

Keywords: giant cells, syncytia, common variable immunodeficiency, multinucleate, monocyte fusion.

Corresponding Author: Tim Scott-Taylor,
Faculty of Life Sciences and Computing
London Metropolitan University
160-220 Holloway Road
London N7 8DB
Telephone 0207 133 4358
E-mail: t.scott-taylor@londonmet.ac.uk
Abstract

Peripheral monocytes from patients with common variable immunodeficiency (CVID) had on average a 2 fold greater tendency to form giant cells in medium without additional cytokines. Giant cell formation was faster and 3 to 5 fold higher in most CVID cells compared to normal. Addition of IL4, GMCSF, IFNγ, TNFa and both T cell and monocyte conditioned media promoted monocyte fusion of some CVID individuals over 5 fold the normal average level, with combinations of cytokines and monokines acting synergistically. The reduction of normal giant cell formation by anti-IFNγ antibody and a greater tendency of CVID cells to fuse in immunoglobulin conditioned media suggests that standard IVIg treatment contributes to granuloma formation. CVID and normal giant cells expressed similar levels of phenotypic molecules and had similar phagocytic activity. Monocytes from many CVID patients have an elevated tendency to fuse which may explain the high incidence of granulomatous complications in CVID.
Introduction

Multinucleated cells are observed regularly in the immune response to malignancy (Cohen and Kurzrock, 2007), and some microbial and parasitic diseases (El-Zammar and Katzenstein, 2007). First reported in patients with tuberculosis over a century ago, multinucleated giant cells are formed by macrophage fusion (Kreipe et al., 1988) and represent an end-point in myeloid cell lineage development. Giant cells are a regular feature of the immune response to diseases characterised by pathogen survival in phagocytes, such as leprosy, syphilis and aspergillosis (Segal et al., 1998); they are also a component of chronic inflammatory reactions to parasites, including schistosomiasis, Leishmania and Filaria (Bentley et al., 1985). Non-caseating epithelioid cell granulomas occur in about 10% of patients with Hodgkin’s lymphoma, and less frequently in other malignant conditions (Brincker, 1986), particularly lung, prostate and some skin tumours (Kornacker et al., 2002; Kurata et al., 2005).

Granulomatous reactions centred round multinucleate giant cells are a pathological feature of a variety of inflammatory diseases, including sarcoidosis, Crohn’s disease and rheumatoid arthritis (Barnard and Newman, 2001). Epithelioid or giant cells, which form the centre of granulomatous reactions, are generated in these diseases from macrophages by the action of inflammatory cytokines (Kasahara et al., 1989). Granulomatous reactions are a recognised feature of common variable immunodeficiency (CVID) with about 20% of patients developing unexplained chronic inflammation, often with granuloma formation, involving many different organs, most commonly the spleen, lymph nodes, liver or lungs (Ardeniz and Cunningham-Rundles, 2009; Bates et al., 2004). Although recurrent bacterial infections due to failure of antibody production is the predominant clinical problem in CVID, the circulating T cells and monocytes in many patients show features of persistent activation (Holm et al.,
2006), with evidence that herpes viruses provide a major inflammatory stimulus in some patients (Marashi et al., 2012). CVID monocytes generally generate defective dendritic cells with deficient major histocompatibility complex (MHC) class II DR expression (Scott-Taylor et al., 2004) which induce poor T cell proliferation and abnormal cytokine production (Bavry et al., 2005; Cunningham-Rundles and Radigan, 2005). Abnormal monocyte responses may contribute to a polarised Th1 immune phenotype in CVID, with exacerbated T cell IL-12 production (Cambronero et al., 2000) and increased IL-12 receptor expression (McQuaid et al., 2000), promoting a high incidence of granulomatous infiltration of lymphoid and other tissues (Webster, 2001). In this study, we have investigated the formation of giant cells from CVID monocytes, which could explain the abnormally high rate of granuloma formation in CVID and elucidate the process of inflammatory polarisation in some CVID individuals.
Materials and Methods

Patients

Twenty five 24 patients (18 females, 6 males, mean age 49yrs, range 26-75 yrs.) attending the Royal Free clinic with a diagnosis of Common Variable Immunodeficiency (CVID) based on IUIS criteria (Conley et al., 1999) (Table 1). Blood samples were obtained prior to immunoglobulin therapy from patients with no clinical symptoms of infection. Healthy adult volunteers (9 females, 7 males, mean age 40yrs, range 22-66yrs) provided blood samples. Patient and control blood donors were healthy and free from overt signs of infection at the time of donation. All donors gave informed consent and the study had local ethical approval (ref: 04/Q0501/119).

Cell Culture

Peripheral blood was collected in lithium heparin tubes, mixed with an equal volume of X vivo 15 medium (Cambrex Bio Science, Wokingham) and mononuclear cells (PBMCs) separated on Lymphoprep (Axis-Shield ProC As, density 1.077 g/ml) after centrifugation. Plasma supernatants were collected, clarified by centrifugation at 500 x g and used to culture autologous cells. PBMCs were washed twice in medium to reduce platelets and then seeded into 6 well plates (Nunc 152795, Sigma-Aldrich, Poole, Dorset). Routine monitoring of immunologic parameters was performed by the Clinical Immunology Service of the Royal Free Hospital using isotype controls in parallel with each batch of samples analyzed. Peptide stimulation of T cells was performed on fresh PBMCs stimulated with 10 mg/mL CMV or EBV peptides or without peptide in the presence of anti-CD28 (10 mg/mL) antibody and brefeldin A (10 mg/mL, Sigma-Aldrich) as previously described (Marashi et al., 2012). PBMCs were stained with the appropriate HLA pentamer (10 mg/mL) for 15 minutes at room temperature, washed with PBS containing 0.1% sodium azide, stained for 15 minutes at room temperature with CD8 FITC and PD-1 APC antibodies (Pharmingen 551347 and 558694), fixed and permeabilized (Fix &
Perm Kit, GAS-002, Caltag Medsystems) and stained with a PE-conjugated antibody to granzyme B (PharMingen, 561142). T cell clonal analysis was performed immediately on a FACSCalibur flow cytometer using a minimum of 50,000 events. Individuals with a >1% total T cell reactivity with CMV tetrameric reagents were designated CMV positive (Table 1).

Adherent monocytes were selected in 2 successive 2 hr. incubations in 6 well plates, from which non adherent cells were carefully washed off and adherent cells eluted by incubation with 5mM EDTA for use in fusion assays. CD14+ monocytes were purified to <98% using an MS column (130-042-201, Miltenyi Biotech, Bisley, Surrey) containing magnetic sheep anti-mouse IgG coated beads (Dynal Biotech, 120-000-305) incubated with 1 ug/ml CD14 antibody (BD PharMingen, San Jose, CA, 550376) for 30 minutes. B lymphocytes (>95% purity) were selected with CD19+ magnetic beads (Miltenyi Biotech,130-052-201) from the non-adherent cell fraction before T lymphocytes (> 98% CD3+ purity by flow cytometry) were separated by 2 rounds of negative selection using a mixed antibody (Miltenyi Biotech, 130-091-156). CD14+ monocytes were cultured in X vivo 15 medium with 10% autologous plasma or 10% heat treated (56°C for 30 min) AB serum (Sera-Lab S-11-J, Haywards Heath, West Sussex) at a density of 5 x10⁵ per ml at 37°C in a humidified atmosphere containing 5% CO₂ with a variety of cytokines and mitogens for 7 days. TNFα, IFNγ or IL1α (5 to 50 ng/ml, Peprotech EC, London) were added to some cultures; some culture wells were coated with 10% BSA or type-1 collagen (Sigma-Aldrich, C3867) in coating buffer (50mM Na₂CO₃, pH = 9.5) overnight at 4°C. Cell viability was monitored by trypan blue exclusion and media including cytokine reagents changed every third day of culture. LPS contamination of media (Limulus Amoeba Lysate kit, Quadratetech Ltd, Epsom, Surrey) was below 15 ng/ml.
T cell conditioned media were generated from normal and CVID T lymphocytes incubated overnight in 6 well plates at a density of $1 \times 10^7$ cells per ml with 1 ug/ml PHA (nPHA and cPHA, respectively) or in wells coated with 20 ug/ml anti-CD3 antibody (nCD3 and cCD3). Antibody coated plates were washed three times with cation-free phosphate buffered saline before use. Normal and CVID monocyte conditioned media was collected from $1 \times 10^7$ pooled purified CD14+ normal and patient cells incubated overnight in 6 well plates coated with 10 mg/mL human gamma globulin (Sandoglobulin, CSL Behring, Haywards Heath) (nIVIG and cIVIG, respectively) or 200 nmol/mL phorbol myristate acetate (Sigma Aldrich) (nPMA and cPMA, respectively). Cell supernatant was clarified by centrifugation for 10 minutes at 500 xg, passed through 0.2 um non-pyrogenic syringe filters and stored at -70°C in 1 ml aliquots. The cell- free conditioned medium was added to monocyte or PBMC cultures at a final concentration of 50% in at least 3 experiments with different conditioned media.

Immature monocyte derived dendritic cells (MdDC) were generated from adherent monocytes after 6 days of culture in X vivo 15 supplemented with 20% autologous plasma, 100 ng/ml GM CSF and 50 ng/ml IL4 (PeproTech EC, London, AF-300-03 and AF-200-04). MdDC were washed and applied to poly-L-lysine (P4832, Sigma-Aldrich, Poole Dorset) treated glass coverslips in 200 ul drops of fresh X vivo 15 at $10^6$ cell/ml in 6 well plates. After 1 hour incubation in a humidified chamber, coverslips were gently submerged in 3 ml of X vivo 15 medium supplemented with 100 ng/ml GM CSF and 50 ng/ml IL4.

The influence of maturation and differentiation agents on giant cell formation was investigated with or without autologous plasma with the addition of cytokines; GM CSF or
G-CSF, (Peprotech EC, AF-300-23, 100 ng/ml), IL4 and TNFα (Peprotech EC, AF-300-01a, 20 ng/ml), IFNγ (5-20 ng/ml), IL1α (10 ng/ml), IL10 (5 and 10 ng/ml) (Peprotech EC, AF-300-02, AF-200-01a and AF-200-10) or mitogens PMA and PHA (2.5-10 ng/ml, P1585 and L2769, Sigma–Aldrich) added at various times to different cultures. CD3 antibody (OKT3 cell supernatant) was used to coat some wells at 5 μg/ml in phosphate buffer overnight prior to the addition of cells. Anti-IFNγ antibody (Remicade, 100 mg/ml) was added to cytokine cultured cells at a concentration of 1 μg/ml for 5 days prior to harvest. Wells were flooded and media exchanged gently by pipette to minimise disturbance throughout. At various time points as described, cells were fixed in ice cold 2% paraformaldehyde PBS for 30 minutes at 4°C and stained with Wright Giemsa stain.

To investigate the effect of differentiation on fusion, cell were preincubated for 15 minutes in X vivo 15 medium without FBS in 10 μM phalloidin FITC, phalloidin oleate and 5 to 40 mM cytochalasin D (Sigma–Aldrich, P5282, P9992 and C2618) prior to washing twice in PBS. incubated in 6 well plates in different concentrations of IL4 and GM CSF between 20 and 100 mM for various times before cells were centrifuged at 180 xg for 10 minutes at 1 x 10^5 cell per ml in 24 well plates over poly L lysine coated coverslips, submerged, cultured overnight, fixed in paraformaldehyde, and stained with Wright Giemsa. Stained cells were examining using a 20x objective lens and an eyepiece graticule, counting the number of nuclei within giant cells containing more than one nucleus and the total number of nuclei in a given area of the cell sheet. The fusion index (FI) was calculated according to the following formula: FI (%) = (number of nuclei within giant cells)/ (total number of nuclei counted) x100. Between 300 and 500 nuclei from selected representative fields were counted for each experiment. Phase contrast and fluorescence photographs were taken with an inverted fluorescence Olympus IMT-2 microscope.
Phagocytosis

Saccharomyces cerevisiae was grown in 10% FBS/RPMI 1640 overnight at 37°C, washed in RPMI, fixed in Leukoperm (Serotec, BUFO9B) and stained with 10 mg/ml ethidium bromide in PBS for 10 minutes. Yeast were washed twice each in DMSO and PBS and resuspended in PBS at 1x 10^8/ml. Yeast and PE conjugated carboxylated beads (6 um diameter, Molecular Probes, Oregon) were added to monocyte cultures on day 6 at a ratio of 10:1 cell. Attached unincorporated beads and yeast were removed prior to FACS analysis by treatment with 0.5% trypsin in PBS at 37°C for 5 minutes or cold DMSO for 5 minutes.

Giant Cell Ultrastructure And Expression

Surface expression of giant cells was examined in cells fixed in 1% PFA/PBS for 15 minutes and permeabilized with 0.01% saponin (Sigma-Aldrich, 47036) in 1% mouse serum/PBS for 15 minutes at 4°C and rinsed twice with excess PBS. Non specific sites were blocked by incubation in 5ug/ml mouse serum/1% BSA/PBS for 30 mi at 37°C before cells were washed and stained with FITC-conjugated mouse anti-human antibodies to MHC II DR, CD83, CD14 (BD Pharmingen 555560, 556855 and 555398, respectively), and CD86 (MCA1118F, Bio-Rad AbD Serotec, Kidlington, Oxford), CD80 AlexaFluor 647 (MCA 2071A Bio-Rad AbD Serotec), CD64 and CD16 PerCP 16 (Pharmingen, 561194 and 5655421) or isotype control antibodies (BD Pharmingen, 345815, 345817) and 1 ng/ml propidium iodide in 0.5 ml. Conjugated F-actin was stained with fluorescein phalloidin (F432, Invitrogen) added at 1.5ug/ml in 0.01% saponin/1% mouse serum /PBS for 30 minutes at room temp. The coverslips were then washed in 0.01% saponin/PBS, again in PBS, and mounted on glass slides in one drop of Dako fluorescent mountant with a spot of no 2 strength nail varnish at each corner of
the coverslip to preserve cell height. Confocal images were acquired on a Zeiss LMI 501 microscope and analysed with BioRad Lasersharp software.

Ultrastructure studies were performed on cells fixed by immersion in 4% glutaraldehyde/2% glutaraldehyde in cold PBS to 15 min. Cells were washed in PBS and placed in 1% osmium tetroxide dissolved in 1 mM cacodylate buffer, pH 7.5 for 1 hr at room temperature. The samples were dehydrated through an increasing ethanol series, staining in 2% uranyl acetate in 30% ethanol in the process and embedded in TAAB 812 resin (TAAB Laboratories Equipment Ltd, Aldermaston). Ultrathin sections were cut on a Leica UCT ultramicrotome and floated onto Formvar-supported grids that were then contrasted with uranyl acetate and lead citrate. Micrographs were taken on Zeiss M-10 electron microscope.

Statistical analysis
Statistical analyses were performed using SPSS for Windows. Levels of cytokines and numbers of cells expressing surface molecules were tested using an independent sample two-tailed t-test (assuming normal distribution of data), and by the Mann–Whitney test for nonparametric data. Spearman’s Rho test was used to assess correlation. P-values < 0.05 were considered significant.
Results

**Morphology And Formation Of Giant Cells**

Culture of PBMCs from either healthy or CVID patients with GM CSF and IL4 over 7 days results in a small proportion of giant cells with multiple nuclei. Characteristics of these pleomorphic cells are shown in Figure 1: they have an extended cytoplasmic border surrounding a central zone containing 2 to 20 nuclei amid dense granular inclusions (Figure 1A). The upper surface was frequently attached to rounded monocyctic cells at various stages of fusion. Nuclei were not incorporated in a symmetrical or uniform pattern but appear to accumulate randomly in a central zone (Figure 1B). In the absence of GM CSF or IL4 purified CD14+ monocytes formed CD68+ dome shaped cells with large central single nuclei in cobblestone formation typical of macrophages (data not shown). CD3+T cells or CD19+ B cells added to CD14+ purified monocytes restored the formation of giant cells but were not themselves incorporated into the giant cells. Monocytes cultured in plastic wells with GM CSF and IL-4 formed multinucleated giant cells but when seeded over a monolayer of epithelial and fibroblast cells (A549, NIH 3T3, 293T, Meso 33), or implanted in an artificial matrix of agar and matrigel, failed to fuse. Coating plates with collagen, BSA or poly-L-lysine increased the fusion rate negligibly (data not shown).

**Kinetics Of Giant Cell Formation In CVID**

When monocytes from CVID patients were cultured with GM CSF and IL4 multinucleated cells were observed after day 2 of culture (Figure 1C), increased till day 5 (Figure 1D) and were generally complete by day 7 of culture. The fusion rate of adherent monocytes was examined in cultured cells from 9 CVID and normal individuals over 9 days of incubation in GM CSF and IL4. Figure 2A shows there was a 2.2 fold average higher number of giant cells at day 9 with CVID monocytes when compare to cells from
healthy controls, although there was wide variation between cells from different patients. Furthermore, the cells from CVID patients formed multinucleate cells with a mean number of 77.3 per 10 high power fields (range 23 to 130) compared with mean of 34.0 (range 13 to 46) nuclei in normal multinucleate cells (p = 0.002; Figure 2A). The rate of cell fusion in CVID cell samples was both faster and greater, resulting in more multinucleated cells that were formed earlier than in cultures of normal cells (Figure 2B). Sequential observation of plates indicated that after day 3 of culture, the increase in multinucleate cell number occurred largely by addition of further cells or by combination of already formed giant cells.

**The Influence Of Plasma, Cytokines And Immunoglobulins On Giant Cell Formation Differed For Normal and CVID Cells.**

The effect of immunoactive agents was evaluated on normal and CVID adherent monocytes (Figure 3A) and CD14+ cells (Figure 3B), seeded in triplicate in 24 well plates at a density of 5x 10^5 /ml, stained in Wrights stain to assess the fusion index on day 7 of culture. The basal rate of giant cell formation in autologous plasma was low in normal adherent monocytes (mean fusion index 2.55 +/- 0.51 standard deviation (mean FI+/-SD) and an average of 1.93 fold higher (4.92 +/- 0.9, mean FI+/-SD) with 9 CVID individuals (Figure 3A), ranging from 0.95 to 3.1 fold higher (P = 0.002). When added in a range of concentrations, a number of inflammatory cytokines and mitogenic stimuli greatly augmented fusion of both normal and CVID PBMCs, but to differing degrees. Several cytokines, including IFNα and IL10 had a negligible effect on the basal rate of multinucleation of CVID and normal cells (n = 3) and data are not shown. Both GM CSF and IL4, individually enhanced fusion indices in normal monocytes (6.8 +/- 1.4 and 8.1 +/- 1.9, mean FI+/-SD, respectively, n = 6), increases of 2.6 and 3.2 fold, respectively, over the normal basal fusion index without reagents. CVID cell fusion indices with GM
CSF and IL4, (9.3 +/- 5.9 and 13.7 +/- 3.7 FI+/SD, respectively, n = 6) were 1.9 and 2.8 fold higher than CVID basal rates. Induction of multinucleate cells by a combination of GM CSF and IL4 was additive, together generating 3.9 and 4.8 fold increases in the fusion index of CVID and normal monocytes (n = 6), respectively, over the basal rates without cytokines. CVID fusion indices with GM CSF and or IL4 were higher than normal cells (P = 0.005) but the induction of cell fusion rates over the basal untreated cell fusion indices by these cytokines concentrations was less powerful for CVID cells than for normal adherent monocytes. TNFα, IFNγ and IL1α were all active in promotion of multinucleate normal cells generating mean fusion indices of 8.7, 9.6 and 8.6 (Figure 3A), respectively, 3.4 fold, 3.8 and 3.4 fold increases, respectively, over the normal basal fusion index without reagents. With CVID cells, TNFα, IFNγ and IL1α, promoted mean fusion indices of 13.6, 16.6 and 5.1, respectively, 2.8 fold, 3.4 and a relatively low 1.04 fold increase for IL1α of multinucleate CVID cells over basal fusion index. In normal cells, both IL1α and TNFα had a synergistic effect with IFNγ, promoting an additional 1.9 and 1.4 fold increase in the fusion index over IFNγ alone whereas in CVID cells (n = 4), TNFα had a minor synergistic effect with IFNγ, promoting an additional 0.4 fold increase in the fusion index over IFNγ alone, but the addition of IL1a alone or when combined with IFNγ did not add to fusion of CVID cells (data not shown).

CVID T Cell Response To Anti-Inflammatory Agents and Mitogens Differed From Normal.

Incubation of cultured cells in anti-TNFα antibody reduced the mean basal fusion index of normal cells by up to a half (mean 51.2%, range 38 to 58%, n = 5) and IL4 +GM CSF cultured cells by more than a third (mean 40.3% reduction, range 31 to 48%). CVID PBMC basal fusion rate was less affected by anti-TNFα antibody (mean 26.8% reduction, range 20 to 29%) than normal cells (P = 0.42) while IL4 + GM CSF cultured
cells fused 24.2% (range 17 to 28%, n = 6) less frequently in anti-TNFα antibody.

Mitogenic stimulation of adherent mononuclear cells with PMA (10 ng/ml) and PHA were highly active in formation of giant cells from normal monocytes (n = 5), while PHA (5 to 20 ng/ml) and CD3 antibody stimulation (data not shown) induced a relatively low response on CVID monocytes.

**Conditioned Media From Purified T Cells And Monocytes Generated Disproportionate Results From Normal and CVID Monocytes.**

Normal CD14+ cells cultured in X vivo 15 medium supplemented with 50% PBS or heat treated AB serum had low fusion indices (0.7 +/- 0.06 and 1.7 +/- 0.6 mean FI +/- SD, respectively, n = 4 normal individuals) with CVID CD14+ cells generating almost 3 fold higher rates of multinucleate cells (2.2 +/- 1.5 and 3.8 +/- 1.2 mean FI +/- SD, respectively, n = 5 CVID individuals), as shown in Figure 3B. Levels of giant cells generated by conditioned media, except those of CVID cells generated with PHA and CD3 antibody, exceeded those of single or combinations of cytokines. T cell conditioned media generated from normal and CVID cells with PMA (nPMA and cPMA) were equally as effective in promoting cell fusion in CVID cells (25.4 +/- 6.3 and 23.7 +/- 8.1 mean FI +/- SD, n = 5 normal cells with nPMA and cPMA, respectively), and also showed approximately equivalent but less activity with normal CD14+ cells (18.8 +/- 1.6 and 17.1 +/- 3.2 mean FI +/- SD, n = 5 CVID individuals, nPMA and cPMA, respectively). CD3 generated conditioned media of normal cells (nCD3, Figure 3B) also had potent effects on the fusion indices of CVID cells and less effect with normal cells (20.6 +/- 4.8 and 14.6 +/- 5.7 mean FI +/- SD, n = 5 normal and CVID individuals, respectively). CD3 generated conditioned media with CVID cells (cCD3) was also relatively more effective with CVID CD14+ cells but was less potent than other conditioned media (12.9 +/- 4.8 and 10.6 +/- 2.4 mean FI +/- SD, n = 5 CVID and normal individuals, respectively).
Conditioned medium generated with PHA from normal cells was highly potent and again relatively more effective with CVID CD14+ cells (nPHA FI +/- SD = 23.4 +/- 4.6 and 17.8 +/- 4.0, n = 6 normal and CVID individuals, respectively) while conditioned medium generated with PHA from CVID cells was much less effective with either normal or CVID CD14+ cells (cPHA FI +/- SD = 11.7 +/- 2.1 and 11.2 +/- 3.1, n = 5 normal and CVID individuals respectively). The use of monocyte media conditioned with gamma globulin also demonstrated a difference between normal and CVID cells. Monocyte conditioned media produced by normal cells (nIVIg) generated only marginally more giant cells than basal levels but media from CVID monocytes stimulated with IVIg generated 3.9 and 4.9 fold increases in fusion index of normal and CVID cells, respectively, (10.3 +/- 2.9 and 14.8 +/- 5.3 mean FI +/- SD, n = 6 normal and CVID individuals, respectively) over plasma alone.

**Giant Cell Function**

Centrifugation of monocytes cultured with GM CSF and IL4 onto coverslips in tissue culture plates promoted formation of very large giant cells. Optimal plasticity in fusion in both normal and CVID cells occurred after 2 to 3 days and before day 5 of culture. The arrangement of nuclei in these large giant cells (Figure 4) was predominantly central but seldom symmetrically or circularly organized. Peripheral nuclei continued to fuse with the main body after the initial fusion event, but newly incorporated nuclei were not repositioned. 1 um confocal optical slices in phalloidin FITC treated cells (Figure 4B) show the nuclei to be scattered in the cytoplasm in an irregular arrangement at varying heights above the substrate. Central nuclei were embedded in an intricate meshwork of F-actin microfibrils, while peripheral nuclei were associated with larger actin microfibers. Preincubation of monocytes in phalloidin oleate reduced syncytial formation and resulted
in widely dispersed nuclei, implicating actin mediated transport in the rearrangement of nuclei in giant cells. Inhibition of syncytia in both CVID and normal cells was also dependent on the concentration of cytochalasin D in the medium; 10 mM cytochalasin D reduced CVID and normal mean cell fusion 3.2 +/- 1.6% and 4.6 +/- 2.3%, respectively, 20 mM cytochalasin D 46.4 +/- 8.8% and 41.1 +/- 7.3%, respectively, and 40 mM cytochalasin D 73.6 +/- 11.3% and 71.9 +/- 12.8%, respectively, from levels without cytochalasin D, in 3 separate experiments. Both normal and CVID giant cells giant cells expressed MHC class II DR, CD80, CD86 and CD64 by immunofluorescence but not CD14, CD16 or CD83. Expression of class II DR was less intense than undifferentiated monocytes stained in equivalent circumstances but no consistent difference were noted in marker expression between CVID and normal giant cells.

Examination of giant cells in transverse electron microscopy (Figure 5A) showed many small dense mitochondria with well-developed cristae amid rough-surfaced endoplasmic reticulum (RER) associated with the nuclei. The cytoplasm was crowded with dense lysosomal bodies, microfibrils, microtubules and lipid droplets mingled with the mitochondria. Granular vesicles were frequently associated with membrane whorls and toroidal structures (Figure 5B), indicating intense membrane reorganization. Golgi apparati, centrosomes and free ribosomes were evident but no structural features particular to either normal or CVID giant cells were discerned.

Giant cells generated in culture were highly phagocytic, engulfing uncoated and opsonized yeast and bacteria. Figure 5C shows a single CVID giant cell with a large number of ingested yeast particles at various stages of digestion. When re-exposed to yeast particles a day or more later, giant cells would incorporate more particles. Both normal and CVID giant cells incorporated sufficient quantities of carboxylated beads to
distort the cellular shape and contents (Figure 5D). Trypsinized bead-treated cells examined by flow cytometry showed an inverse relationship between the numbers of nuclei and the numbers of beads incorporated (data not shown) but it was not possible to demonstrate a difference in phagocytic capacity of normal and CVID monocytic syncytia with fluorescent bacteria, opsonized or uncoated yeast or carboxylated beads.
Discussion

Giant cells are formed from monocytes in response to a variety of stimuli and in certain inflammatory diseases (Heresbach et al., 2005; Mizumo et al., 2000). Formation of multinucleate cells from peripheral monocytes in vitro is related to granulomatous disease in sarcoid (Mizumo et al., 2001). The tendency of untreated PBMC from CVID individuals to form giant cells in culture was almost twice (1.93 fold on average) that of normal cells. Cell fusion varied greatly between CVID individuals with approximately half of the CVID samples examined forming giant cells at a rate within the normal range and others demonstrating a capacity four or more fold higher than normal cells. Higher fusion indices were not closely associated with CVID classification or antibody concentration and were related to B and T cell concentration. Chronic inflammation in CVID has been associated with low T cell counts and is related to a deficiency in regulatory T cells (Fevang et al., 2007) while those CVID individuals with reduced CD19+ cells and the lowest switched B cell memory cell counts are most prone to autoimmune phenomena and granulomatous disease (Yong, Orange and Sullivan, 2010).

Phenotypic markers of giant cells were indistinguishable in normal or CVID giant cells and similar to monocyte-derived DCs but lacked CD83 expression. Multinucleate epithelioid cells in granulomatous centres similarly contain large amounts of granules, mitochondria, smooth endoplasmic reticulum and free ribosomes (Kirkpatrick, Curry and Bisset, 1988) indicating active metabolic capacity. Frequently observed membrane complexes have been associated with reorganisation and are consistent with plasma membrane vesicle reduction in fused cells. Both normal and CVID giant cells were phagocytic and capable of killing engulfed bacteria and yeast cells. The inverse relationship between the numbers of nuclei and the numbers of beads incorporated by phagocytic giant cells suggests that more mature giant cells were proportionally less
phagocytic than monocytes. Phalloidin staining showed centralised nuclei embedded in a diffuse network of actin microfilaments whereas peripheral nuclei were often linked to larger bundles of fibres. Phalloidin oleate, a cell permeable inhibitor of stable actin formation, and cytochalasin D which affects actin filament elongation (Bear et al., 2002) prevented nuclear aggregation and reduced the fusion index, suggesting that actin microfilament elongation enabled nuclear reorganisation in giant cells and has a role in cell fusion.

CVID giant cells had higher number of nuclei and were formed earlier in culture than normal giant cells. Accumulation of multicellular nuclei in later stages of culture occurred largely by amalgamation of adjacent giant cells or further incorporation into established giant cells rather than continued fusion between single cells, implying that a limited number of cells in culture were capable of fusion in vitro and that these cell continued to be active in fusion at subsequent times. IL4 and GM CSF concentrations suitable for generating monocyte derived DC, boosted the fusion rates 2 to 3 fold and acted additively (Dugast, Gaudi and Toujas, 1997), augmenting the formation of giant cells up to 5 fold over the basal rates in both normal and CVID cells. Inflammatory lymphokines, particularly IFN_γ and TNFα, dramatically induced the fusion rates in both normal and CVID monocytes, in keeping with previous findings that IFN_γ is pivotal in giant cell formation (Most et al., 1990; Fais et al., 1994). IFN_γ also acted additively with other cytokines in promoting syncytia (Byrd, 1998); combinations of IFN_γ with IL4 or GM CSF generating the highest fusion indices, over 5.5 or 6 fold the unstimulated monocyte fusion rates in CVID and normal cells, respectively, indicating that monokines and lymphokines can cooperate in monocyte fusion in normal and CVID cells alike. The reduction in giant cell formation in some CVID cell samples the presence of IL10, which acts to reduce cytokine synthesis by monocytes (de Waal et al., 1991), would suggest that monocyte cytokine production can
promote granuloma in CVID as supported by the potent fusogenic activity of CD14+ cell conditioned media. T cell mitogens were also potent inducers of normal as well as CVID fusion of monocytes and the equivalent activity of T cell and monocyte conditioned media in generating fusion of purified CD14 cell populations would indicate that both lymphokines and monokines are both sufficient to generate fusion. Similarly, supernatants from mitogen-stimulated PBMC were the major stimulus to fusion of normal monocytes (Postlethwaite et al., 1982) and were as effective inducers of cellular fusion as optimized cytokine concentrations when added to monocytes isolated from individuals with sarcoid (Most et al., 1997). It is likely that monocytes and lymphocytes, and possibly other cells in vivo, produce cytokines that synergise in promoting granuloma formation. Th1 cells, producing INFγ (Wahlstrom et al., 2001), probably aggregate in response to IL12 production by APCs (Rissoan et al., 1999), leading to TNFα production (Grunewald and Eklund, 2007) and further promotion of IL12 production in a pro-inflammatory loop in granulomatous foci. The importance of IFNγ in T cell recruitment and macrophage activation has been demonstrated in a mouse model where deficiency in IFNγ production prevents granuloma formation (Pearl et al., 2001). CVID T cells synthesize more IFNγ and CVID monocytes higher amounts of IL12 than healthy individuals when stimulated with LPS (Cambronero et al., 2000), contributing to a Th1 cytokine milieu that would mitigate against B cell class switching and antibody production.

Higher numbers of multinucleate cells and more nuclei per giant cell were generated in the CVID cell samples. However, the basal fusion index of untreated normal PBMC was augmented more by addition of IFNγ, TNFα and T cell mitogens than was the basal fusion rate of CVID cells. These observations would suggest that activated CVID peripheral monocytes (Scott-Taylor et al., 2006; Barbosa et al., 2012) are less amenable to further cytokine induction in vitro. The comparatively low stimulus to CVID cell fusion
relative to normal cells of IL1α and PHA conditioned media may instead be inherent to an early T cell signal transduction defect in immunodeficiency (Zielen et al., 1994; Fiedler et al., 1987). The greater reduction of the cytokine induced fusion rate for normal than CVID cells by incubation in anti-TNFα antibody would indicate that monocyte fusion partially acts through TNFα and that activation of monocytes in CVID is influential in giant cell formation. Elevated serum TNFα levels in CVID are related to low CD4+ T cell counts (Aukrust et al., 1996) and to autoimmune phenomena (Knight and Cunningham-Rundles, 2006). A high proportion of CVID individuals with granulomatous disease have a TNF receptor mutation that may predispose to inflammatory disease (Mullighan et al., 1997). Anti-TNFα treatment can reduce granulomatous lesions in CVID (Lin et al., 2006; Thatayatikom et al., 2005) and classical sarcoid disease (Wells et al., 2008). In vitro anti-TNFα antibody had little effect on the basal monocyte fusion rate but did particularly affect the fusion index of CVID cells treated with IL4 and GM CSF. The fusion of normal monocytes was inhibited more by anti-TNFα treatment than CVID cells, indicating that the constitutional activation of CVID peripheral monocytes may make them unamenable to anti-TNFα antibody treatment and that other factors are involved in monocyte fusion in CVID.

The promotion of in vitro fusion by conditioned media of monocytes treated with gamma globulin raises the question of whether this standard treatment of CVID may increase the risk of granulomatous disease, possibly by enhancing FcγRI expression. IVIg therapy predominantly reduces inflammatory responses (Sewell et al., 1999; Ballow et al., 1989) and suppressed the production of IFNγ and IL12p70 from B cells (Bayry et al., 2011). Nevertheless, increased TNFα and TNFα receptor expression has been documented to IVIg therapy in IgG deficient (Farber et al., 1994) and CVID individuals (Sewell et
al., 1999) and correlated with adverse inflammatory reactions (Aukrust et al., 1994). IVIg infusion increased CD64 expression in monocytes (Ling et al., 1993) through inflammatory cytokine expression and could contribute to the occurrence of granulomatous complications in CVID by promotion monocyte fusion through Fc receptor interaction. In summary, this data provides further evidence of a disturbed myeloid lineage in CVID that may contribute to pathology in immunodeficiency. Besides the well described defects in circulating T cells, constitutional activation of peripheral monocytes may explain the predisposition of CVID patients to chronic inflammatory complications including granuloma.

Acknowledgements
We thank Sister Cilla Freud and Sari Workman for great assistance with samples. David Guzman was very helpful in the definition of patient characteristics. The work was part funded by a European Union grant (IMPAD-QLRT-2001-01536).
References


Knight AK, Cunningham-Rundles C. Inflammatory and autoimmune complications of common variable immune deficiency. Autoimmunity Reviews 2006; 5,156–159


Figure Legends.

Figure 1: Appearance Of Multinucleate Cells In Culture. Large multinucleate cells appeared in culture of adherent monocyctic cells in GM CSF and IL4 (A); in refracted light at x20 magnification. (B): stained with MHC class II DR FITC and DAPI. CVID monocyctic cells PBMC were culture in 24 well plates and stained with Wright’s stain at various times. Fused cells with multiple nuclei were evident by day 3 (C) and well advanced by day 7 (D) of culture.

Figure 2: Comparison of Multinucleate Cell Formation In CVID and Normal Individuals. PBMC plated in 6 well plates at a density of 5 x 10^5 cells per ml in GM CSF and IL4 and examined under a bright field microscope. Cell fusion was enumerated in 10 high power fields. Data displayed as A: mean ( ), 25 to 75% (□ ) and complete range ( ) of the fusion index of normal and CVID cells of 9 individuals. B: individual fusion rates of CVID patients ( X and dashed lines ) overlap with the fusion rate of normal individuals ( mean multinucleate cells of nine individuals +/- standard deviation, complete lines ) but normal fusion rates were lower and more consistent. Statistical evaluation was performed by Student T test.

Figure 3: Multinucleate Cell Formation in Normal and CVID Cells To A Variety Of Stimuli. A: Peripheral blood mononuclear cells were cultured for 7 days in a range of concentrations of cytokines and mitogens or with anti-TNFα antibody and the fusion index generated as (the number of nuclei in multinucleate cells) / (total number of nuclei counted) x100. Results are expressed as the mean +/- the standard deviation of 3 or more individuals. PBMC of CVID patients had a higher capacity to generate multinucleate cells to different stimuli and growth conditions than those of healthy controls. B: CD14+ cells were cultured in X vivo 15 medium supplemented with PBS, or pooled human AB serum,
or conditioned medium from normal or CVID cells treated with OKT3 antibody (nCD3 and cCD3) or stimulated with PMA (nPMA and cPMA) or PHA (nPHA and cPHA) or pooled human immunoglobulin (nIVIg and cIVIg). •• = normal cells, ••• = CVID cells. Fusion indices represent 3 or more separate experiments. Statistical values generated by Mann Whitney U test. *P = 0.05, **P = 0.01.

Figure 4: Arrangement of Nuclei In Giant Cells. A: stained with phallolidin FITC and DAPI on day 7 of culture after induction of cell fusion of CVID PBMC by centrifugation of IL4 and GM CSF incubated cells, B: 1 um optical slices of the same cell showing a central nuclei mass surrounded by an intricate microfibril mesh.

Figure 5: High Magnification Examination of CVID Giant Cells. A: Transmission electron micrograph of a giant cell (x5390) illustrating mitochondria (M), dense granules (G) and rough endoplasmic reticulum (ER) closely associated with nuclei (N). Scale bar, 5 um. B: detail of cytoplasm (x23400) of a CVID giant cell showing numerous mitochondria (M), endoplasmic reticulum (ER) and free ribosomes (R). The toroidal structures (arrow) indicate active membrane reorganisation. Scale bar, 500 nm. C: Electron micrograph of a giant cell with numerous yeast cell bodies (arrow) at various stages of digestion. D: confocal microscope illustration of a CVID ingested carboxylated beads (arrow). Fluorescent latex beads of 5 um in diameter were added on day 7 for 4 hours to monocytes cultured at a density of 10^6 cell per well in a 24 well plate in 100 mM GM CSF and IL4 prior to fixation and staining. Numerous beads (arrow) show up as regular array.
Table 1: Patient Immunoglobulin and Lymphocyte Phenotype.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>IgG mg/dl</th>
<th>IgA mg/dl</th>
<th>IgM mg/dl</th>
<th>CD3+ x10^9/L</th>
<th>CD19+ x10^9/Lo</th>
<th>Fusion Index</th>
<th>CMV status</th>
<th>Clinical Presentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>48</td>
<td>0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>2.04</td>
<td>0.03 (1a)</td>
<td>37.3</td>
<td>+</td>
<td>bronchiectasis</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>56</td>
<td>1.5</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>1.93</td>
<td>0.03 (1b)</td>
<td>49.2</td>
<td>+</td>
<td>bronchiectasis, enteropathy</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>75</td>
<td>1.6</td>
<td>0.3</td>
<td>0.2</td>
<td>0.84</td>
<td>0.05 (1a)</td>
<td>52.6</td>
<td>+</td>
<td>granulomatous liver disease</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>63</td>
<td>1.0</td>
<td>&lt;0.1</td>
<td>0.7</td>
<td>1.05</td>
<td>0.22 (1b)</td>
<td>16.3</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>37</td>
<td>0.1</td>
<td>0.1</td>
<td>0.2</td>
<td>1.98</td>
<td>0.23 (1b)</td>
<td>11.5</td>
<td>-</td>
<td>bronchiectasis, enteropathy</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>61</td>
<td>0.6</td>
<td>&lt;0.1</td>
<td>0.15</td>
<td>1.29</td>
<td>0.24 (1a)</td>
<td>17.7</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>55</td>
<td>2.2</td>
<td>0.98</td>
<td>0.41</td>
<td>2.5</td>
<td>0.28 (1a)</td>
<td>18.6</td>
<td>+</td>
<td>bronchiectasis, enteropathy</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>26</td>
<td>3.7</td>
<td>&lt;0.1</td>
<td>0.4</td>
<td>0.94</td>
<td>0.28 (1b)</td>
<td>16.7</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>43</td>
<td>2.9</td>
<td>0.1</td>
<td>0.6</td>
<td>1.54</td>
<td>0.29 (1a)</td>
<td>14.5</td>
<td>-</td>
<td>granulomatous liver disease</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>42</td>
<td>4.4</td>
<td>0.1</td>
<td>0.2</td>
<td>1.38</td>
<td>0.29 (1b)</td>
<td>10.8</td>
<td>+</td>
<td>bronchiectasis</td>
</tr>
<tr>
<td>11</td>
<td>F</td>
<td>52</td>
<td>8.8</td>
<td>0.1</td>
<td>0.4</td>
<td>0.72</td>
<td>0.35 (1b)</td>
<td>26.1</td>
<td>nd</td>
<td>bronchiectasis</td>
</tr>
<tr>
<td>12</td>
<td>F</td>
<td>40</td>
<td>2.9</td>
<td>0.42</td>
<td>1.0</td>
<td>1.62</td>
<td>0.36 (1b)</td>
<td>11.3</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>M</td>
<td>48</td>
<td>3.3</td>
<td>&lt;0.1</td>
<td>1.12</td>
<td>0.76</td>
<td>0.38 (1b)</td>
<td>16.2</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>F</td>
<td>70</td>
<td>3.8</td>
<td>0.5</td>
<td>4.6</td>
<td>1.28</td>
<td>0.5 (1b)</td>
<td>12.8</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>F</td>
<td>52</td>
<td>1.1</td>
<td>&lt;0.1</td>
<td>0.2</td>
<td>1.69</td>
<td>1.02 (1a)</td>
<td>48.6</td>
<td>-</td>
<td>granulomatous disease, bronchiectasis</td>
</tr>
<tr>
<td>16</td>
<td>F</td>
<td>59</td>
<td>0.64</td>
<td>&lt;0.1</td>
<td>0.4</td>
<td>2.65</td>
<td>1.11 (1a)</td>
<td>37.4</td>
<td>+</td>
<td>granulomatous liver disease</td>
</tr>
<tr>
<td>17</td>
<td>F</td>
<td>41</td>
<td>0.9</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>1.40</td>
<td>nd</td>
<td>34.5</td>
<td>+</td>
<td>bronchiectasis</td>
</tr>
<tr>
<td>18</td>
<td>M</td>
<td>49</td>
<td>0.8</td>
<td>0.8</td>
<td>&lt;0.1</td>
<td>0.78</td>
<td>0.003 (1b)</td>
<td>21.3</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>F</td>
<td>51</td>
<td>0.8</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>1.01</td>
<td>0.02 (1b)</td>
<td>24.7</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>F</td>
<td>56</td>
<td>1.4</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>1.75</td>
<td>0.33 (1a)</td>
<td>18.6</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>F</td>
<td>52</td>
<td>0.9</td>
<td>&lt;0.1</td>
<td>0.4</td>
<td>0.73</td>
<td>0.16 (1b)</td>
<td>23.1</td>
<td>+</td>
<td>bronchiectasis, enteropathy, granuloma</td>
</tr>
<tr>
<td>22</td>
<td>M</td>
<td>22</td>
<td>0.8</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>0.55</td>
<td>0.05 (1a)</td>
<td>15.8</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>F</td>
<td>42</td>
<td>1.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>1.65</td>
<td>nd</td>
<td>41.0</td>
<td>+</td>
<td>bronchiectasis</td>
</tr>
<tr>
<td>24</td>
<td>F</td>
<td>31</td>
<td>2.9</td>
<td>0.2</td>
<td>0.5</td>
<td>1.4</td>
<td>0.35 (1a)</td>
<td>38.9</td>
<td>+</td>
<td>granulomatous liver disease</td>
</tr>
<tr>
<td>R²</td>
<td></td>
<td></td>
<td>0.023</td>
<td>0.046</td>
<td>0.13</td>
<td>0.022</td>
<td>0.012</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
<td>0.9</td>
<td>0.83</td>
<td>0.53</td>
<td>0.91</td>
<td>0.96</td>
<td>0.28</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Freiberg classification.  • R² correlation to fusion index.

^P value for unpaired T test comparing fusion indices of CMV positive and negative patients
Fig. 2
Fig. 3
Fig. 4
Fig. 5
Highlights

- The optimal generation of giant cells is described.
- Monocytes from CVID have elevated fusion rates.
- Fusion indices of monocytes of CVID individuals was not related to CMV status, T or B cell numbers or immunoglobulin concentrations.
- Anti TNFa treatment did not reduce giant cell formation in CVID monocytes but immunoglobulin conditioned monocyte media did promote giant cell formation.
- Monocyte disturbances and possibly standard treatment via IVIg contribute to inflammatory pathology in CVID.